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Reactivity of Sulfhydryls in Several β-Lactoglobulins[1]

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Native cow β-lactoglobulins A and B, and goat and sheep β-lactoglobulins reacted with Ellman's reagent at identical rates, and all exhibited two sulfhydryl groups per mole (36,600 gm). Cow β-lactoglobulin C also contained two sulfhydryls per mole, but these reacted at only about one-tenth the rate of those in the other variants. The reactivity of the two sulfhydryl groups in a given variant appeared identical. Enzymic removal of the two carboxyl terminal residues of β-lactoglobulin B greatly increased the reactivity of the sulfhydryls to a rate comparable to that in the molecule denatured with sodium dodecyl sulfate. Denaturation, as measured by rate of reaction of sulfhydryls with Ellman's reagent, required a higher concentration of sodium dodecyl sulfate for β-lactoglobulin C than for β-lactoglobulin A, and least for β-lactoglobulin B.

The genetic variants β-A and β-B[2] of cow β-lactoglobulin were discovered by Aschaffenburg and Drewry (1), and β-C by Bell (2). A comparison of the chemical properties and amino acid composition of the three variants has been made by Kalan *et al.* (3). Goat and sheep β-lactoglobulins have been shown to be homologous to cow β-lactoglobulin by similarity of preparation (4–6), amino acid composition (4, 7), and cross-reactivity as antigens (8). Modified β-lactoglobulins have been crystallized after removal of the C-terminal -His-Val (goat), -His-Ile (cow), and -His-Leu (sheep) by treatment with carboxypeptidase A (9).

The number of sulfhydryl groups in cow β-lactoglobulin has been determined by a variety of methods by Fernandez-Diez *et al.* (10), who found approximately two groups per mole of native protein (36,600 gm). However, these authors (10) who reported the use of DTNB, as developed by Ellman (11), on a sample of β-AB, found 1.1 sulfhydryls per mole of native protein, and 1.9 sulfhydryls per mole of protein denatured in 0.5% SDS. Zak *et al.* (12), using 4-(p-hydroxybenzeneazo)phenylmercuriacetate, found 1.03 sulfhydryls per mole of native protein, 1.78 per mole of protein denatured in 8 M urea, and 2.0 per mole of protein in 1% LiBr. These results suggested either that the two sulfhydryls of the β-lactoglobulin molecule are available to different degrees, or that the sulfhydryls of β-A and β-B react differently.

The rate of reaction of sulfhydryl groups has been used to detect conformation differences between proteins. Guidotti (13) attributed the difference in velocity of reac-

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[2] Abbreviations used: β-AB, β-A, β-B, and β-C, cow β-lactoglobulins AB, A, B, and C, respectively; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); SDS, sodium dodecyl sulfate; EDTA, disodium ethylenediaminetetraacetate.

tion of the sulfhydryls in oxyhemoglobin and carbon monoxyhemoglobin with DTNB to differences in the conformation of the two proteins. Dunnill and Green (14) followed the N $\rightleftharpoons$ R conformation change of β-A by measuring differences in the rates of reaction of *p*-chloromercuribenzoate with the protein over the pH range of 2.43–8.50.

The interaction of detergent, sodium *n*-octylbenzene-*p*-sulfonate, with β-AB has been studied by Hill and Briggs (15). Their adsorption isotherms led to the hypothesis that initially the protein adsorbs a finite number of detergent molecules, calculated as 2.5 per molecule of protein. As the equilibrium detergent concentration is increased, the protein "unfolds" in an all-or-none reaction, the "unfolded" form not being interconvertible with the "native" form. Anderson (16) studied the interaction of sodium *n*-octylbenzene-*p*-sulfonate with β-A and β-B by equilibrium dialysis techniques. He found that β-A and β-B begin to "unfold" at about the same free detergent concentration, but that β-B is almost completely "unfolded" at a free detergent concentration less than that required for β-A. The results suggested that β-A binds 2 moles of detergent in the "unfolded" state, and β-B binds three.

The purpose of the experiments described here was threefold: to determine the number of sulfhydryl groups in several β-lactoglobulins, to obtain evidence for conformational differences between these β-lactoglobulins, and to detect conformation changes resulting from either removal of the two C-terminal amino acids or from the addition of graded amounts of detergent. The parameter measured was the rate of the reaction with DTNB.

MATERIALS AND METHODS

Materials. For all experiments except those involving graded detergent concentrations a stock potassium phosphate buffer of pH 7.6, 0.05 M, was used. The working buffer was made by diluting the stock buffer fivefold with deionized, distilled water and adding 10 ml of a 0.02 M EDTA solution to each 200 ml. For the experiments conducted with graded detergent concentrations, the stock was a sodium phosphate buffer of pH 7.6, 0.05 M, and the working buffer was prepared as described above. Ellman's reagent was prepared by dissolving 40 mg of DTNB (K and K Laboratories) in 10 ml of the desired stock buffer, and was kept refrigerated in a dark bottle.

β-A and β-B were crystallized by the method of Aschaffenburg and Drewry (5) from typed milks. β-C was prepared according to Kalan *et al.* (3), the modified β-lactoglobulins according to Greenberg and Kalan (9), and goat β-lactoglobulin according to the method of Askonas (17). Sheep β-lactoglobulin (4) was obtained from Dr. J. L. Maubois (Ecole Nationale d'Agronomie, Rennes, France). β-A containing 2 moles of SDS was prepared as described by McMeekin *et al.* (18); the SDS bound was determined by the sulfur content of the crystalline compound. The protein solutions were prepared by dissolving 3.7 mg of protein per milliliter of the working buffer. Concentrations were measured spectrophotometrically on a Beckman model DU spectrophotometer.

All detergent solutions were prepared from a 1% stock solution of SDS (Matheson, Coleman and Bell) in the sodium phosphate working buffer. This was prepared fresh weekly. Dilutions were made with the working buffer.

Methods. The reaction with DTNB was conducted in round cuvettes, 19 × 105 mm, in the dark. Optical density was read at 412 mμ on a Beckman model B spectrophotometer at various time intervals. To 7.0 ml of the appropriate working buffer was added 2.0 ml of the protein solution. At zero time the DTNB was added (0.2 or 0.5 ml, respectively, if approximately twofold or fivefold excess reagent was desired), the contents of the tube were mixed rapidly, and the absorbance was recorded at intervals, against the appropriate blank. A standard glutathione curve was obtained with each determination. Where detergent was used, the protein solutions were added to the detergent-containing buffer one hour before the addition of DTNB.

RESULTS

In the first experiment (Fig. 1) the reaction rates of β-A, β-B, β-C, and goat and sheep β-lactoglobulins were compared. Approximately twofold excess reagent was used. Reaction end points were checked by observing optical density up to 10 hours after the reaction appeared to be completed. The results show that the sulfhydryl groups of cow β-A, β-B and goat and sheep β-lactoglobulins react with DTNB at about the same rate, and β-C at a far slower rate.

The second experiment examined the effect of modifying the native protein by removal of the C-terminal sequence -His-Ile

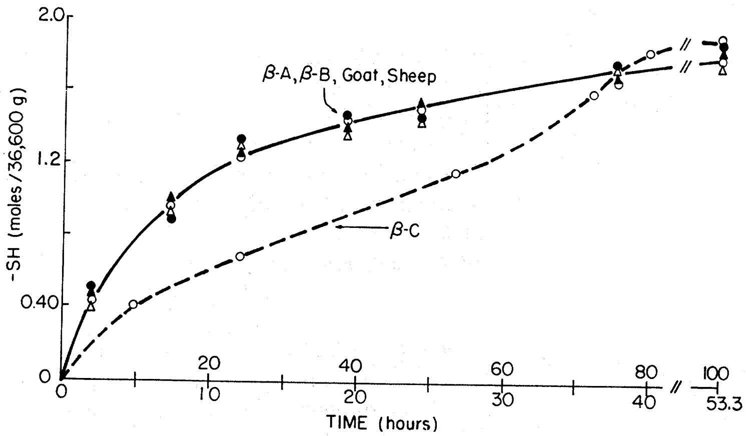

FIG. 1. Reaction of several β-lactoglobulins with twofold excess DTNB in potassium phosphate buffer, pH 7.6, 0.01 M, containing 1×10^{-3} M EDTA (- - -) Top time scale; (——) bottom time scale.

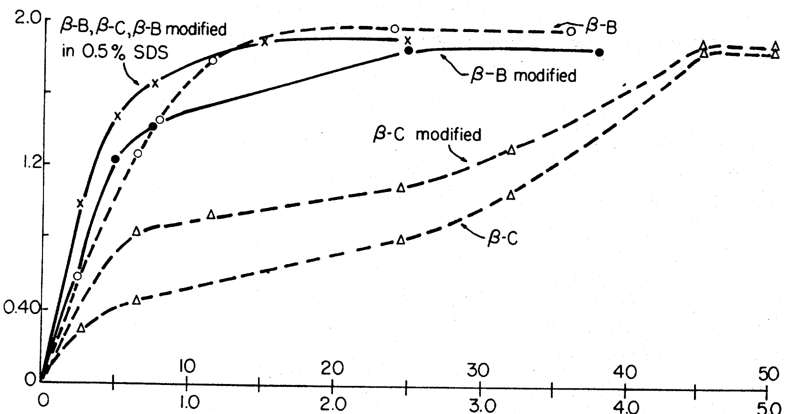

FIG. 2. Reaction of several β-lactoglobulins with fivefold excess DTNB in the presence and absence of 0.5% SDS. Conditions the same as in Fig. 1. (- - -) Top time scale; (——) bottom time scale.

on the rate of reaction of DTNB with cow β-B and β-C sulfhydryl groups. In addition, the effect of 0.5% SDS was examined. Approximately fivefold excess reagent was used, and the reactions were shown to be pseudo-first-order. Acrylamide gel electrophoresis had shown (9) the modified β-B to be homogeneous; however, the modified β-C was observed to consist of a mixture of the native and modified proteins (9).

The results (Fig. 2) show that the modified β-B reacted faster than β-B, and the modified β-C faster than β-C. In 0.5% SDS the denatured proteins, β-B, modified β-B, and β-C react approximately ten times faster than native β-B. The presence of detergent appears to have only a slight effect on the rate at which modified β-B reacts. That the modified β-C is actually a mixture of the modified and native proteins is supported by the fact that the initial reaction rate is similar to that of modified β-B, but its rate decreases with time to parallel that of β-C. The sulfhydryl groups of the modified proteins are more readily available for reaction than those of the native; in fact, they are almost as reactive as those of the denatured proteins.

The effect of concentration of detergent

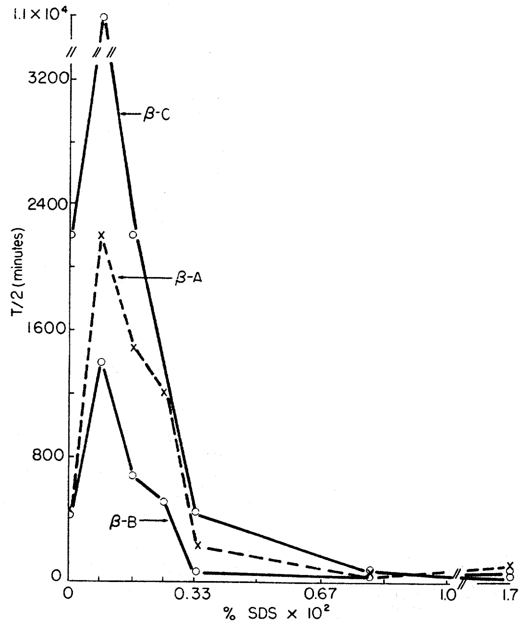

FIG. 3. Reaction half-times of β-A, β-B, and β-C with fivefold excess DTNB, as a function of the percentage of SDS in the reaction mixture; sodium phosphate buffer, pH 7.6, 0.01 M, containing 1×10^{-3} M EDTA.

on extent of denaturation of β-A, β-B, β-C, and the modified variants was examined. Pseudo-first-order reactions were observed with fivefold excess reagent. Reaction half-times were then calculated for each concentration of detergent used, and the results are summarized in Fig. 3. It can be seen that β-B is most susceptible to denaturation by detergent, and β-C least susceptible. The modified proteins followed the same order of susceptibility to denaturation as shown in Fig. 4. β-A containing 2 moles of bound SDS had a reaction half time of 2500 minutes.

DISCUSSION

Native β-A, β-B, and goat and sheep β-lactoglobulins react with DTNB at the same rate, and each contains 2 moles of sulfhydryl per 36,600 gm. No difference in reactivity between the two sulfhydryls of each β-lactoglobulin molecule was detectable by this method; a smooth curve was obtained under conditions in which the reaction followed first-order kinetics. The failure of Zak *et al.* (12) to obtain 2 moles of sulfhydryl per mole of native cow β-lactoglobulin may be due to their use of a commercial preparation of β-lactoglolbuin. The results obtained in this paper differ also from those obtained by Fernandez-Diez *et al.* (10), who found one sulfhydryl per mole of native cow β-lactoglobulin with DTNB. Their results may perhaps be explained by failure to allow the reaction to go to completion.

The similar availability of the sulfhydryls of β-A, β-B, and goat and sheep β-lactoglobulins suggests that the gross conformations of these β-lactoglobulins are similar. The slower reaction rate of β-C suggests that it alone has a different three-dimensional conformation, making its sulfhydryls less available for reaction. Kalan *et al.* (3) found β-C to be more resistant to hydrolysis with carboxypeptidase than were β-A and

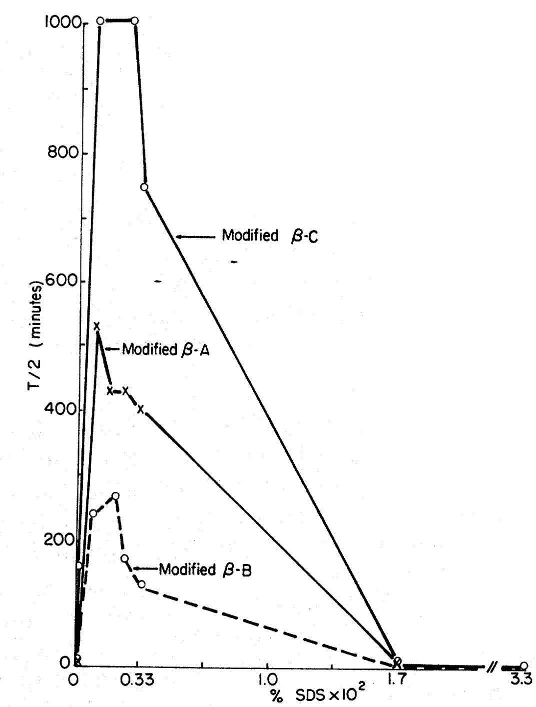

FIG. 4. Reaction half-times of the modified proteins, β-A, β-B, and β-C, with fivefold excess DTNB, as a function of the percentage of SDS in the reaction mixture. Conditions are the same as in Fig. 3.

β-B, and they postulated that this represents a difference in tertiary structure.

The rate of reaction of modified β-B with DTNB is approximately the same as that of native and modified β-B in 0.5% SDS. This suggests either that the modified protein is "denatured," or that the presence of the C-terminal -His-Ile sequence blocks the approach of the DTNB to the sulfhydryl groups of the native molecule. Kopfler and McMeekin (19) found that the solubilities of β-A and β-B are reduced upon treatment with carboxypeptidase A. While removal of the C-terminal -His-Ile sequence affects solubility, this does not necessarily indicate that a conformational change has taken place. Greenberg and Kalan (9) found that the modified proteins have optical rotation values only slightly higher than those of the native proteins, which suggests that the modified materials are not denatured and retain a configuration similar to that of the native proteins. These authors also found the values for the optical rotatory dispersion parameters, a_0 and b_0, of the modified and native proteins to be the same, and concluded that the native and modified proteins do not differ appreciably in secondary structure.

The interpretation of the effects of graded detergent concentrations on β-A, β-B, and β-C was suggested by Anderson (16). His equilibrium dialysis studies on β-A and β-B with sodium n-octylbenzene-p-sulfonate led to the proposal that initially β-A and β-B bind a finite number of detergent molecules. The results reported herein suggest that the β-lactoglobulin variants bind a finite number of SDS molecules initially, and that these bound detergent molecules either block the accessibility of the sulfhydryls to the DTNB molecules, or that the binding of the detergent molecules increases the compactness of the entire protein. Crystallized β-A containing 2 bound moles of SDS per mole of protein had a reaction half-time slightly higher than the maximum found by adding graded concentrations of detergent to β-A. As more detergent molecules are bound, a new electrophoretic form of the protein appears (16); in this region of detergent concentration (about 5×10^{-4} molar equilibrium concentration) the protein is postulated to "unfold" in an "all-or-none" fashion. In the present work the increase in rate of reaction of sulfhydryls with DTNB occurred at a total detergent concentration in this same range; since the protein concentration was only 0.2×10^{-4} M, the free and total detergent concentrations would not differ greatly. Definitive comparisons of the two experiments are probably not justified because of the difference in kind of detergent and pH (pH 6.8 vs. 7.6). Nevertheless the two phenomena—increased electrophoretic mobility and increased reactivity of sulfhydryls—occur in a comparable range of detergent concentration and appear to be manifestations of the same structural change brought about by the interaction of the detergent with the protein.

The greater susceptibility of β-B to denaturation by detergent is a measure of the fact that the β-B molecule is "unfolded" at a lower detergent concentration than that required for β-A. In this connection it is interesting to note that Gough and Jenness (20) found β-B to be more susceptible to heat denaturation than β-A. At detergent concentrations greater than 0.5%, reaction half-times begin to increase somewhat. This is perhaps the result of detergent micelle formation around the protein molecule.

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